Supporting Information

Discovery of CX-5461, the First Direct and Selective Inhibitor of RNA Polymerase I, for Cancer Therapeutics

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Biological assays

***Cell lines*:** All cell lines were purchased from American Tissue Culture Collection (Manassas, VA).

qRT-PCR Assay for Selective Inhibition of RNA Polymerase I transcription

The Pol I transcription assay was developed to measure the compound-dependent inhibition of the synthesis of rRNA versus mRNA. Briefly, this procedure utilizes a quantitative real time polymerase chain reaction assay (qRT-PCR) to quantify the amount of newly synthesized rRNA and mRNA in cancer cells treated with the drugs. The format of this assay is the same for all cell lines tested.

Assay protocol

3000 cancer cells in 100 uL of cell culture media were plated in each well of a 96-well clear bottom, black wall cell culture-pretreated plate. The next day compounds are serially diluted (5-fold in cell culture media) across a 96-well polypropylene mother plate from row A to row F, to yield 6

concentrations (25 uM, 5 uM, 1 uM, 200 nM, 40 nM and 8 nM) for each test compound. Rows G and H contain only DMSO. Once titrations are made, the media in plates with cells were disposed and 100 μL of drug dilutions are transferred to plates with cells. After two-hour incubation at 37 *C, the media with drug dilutions is disposed, the cells in the plate are washed three times with 100 uL of ice-cold PBS and the total RNA from cells is isolated using RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol) and its concentration was determined using Ribogreen reagent (Invitrogen, Carlsbad, CA). Relative levels of 45S pre-rRNA and c-myc mRNA were measured using Applied Biosystems' (Foster City, CA) proprietary primers-probe set for c-myc mRNA and custom primers-probe set (forward primer: CCGCGCTCTACCTTACCTACCT, reverse primer: GCATGGCTTAATCTTTGAGACAAG, probe: TTGATCCTGCCAGTAGC) for pre-rRNA. Analysis was run on 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA).

Cell-free Pol I transcription assay

To measure the direct effect of drugs on RNA Polymerase I transcription, we developed a nuclear extract-based assay.

Assay protocol

Compounds are serially diluted (5-fold in cell culture media) across a 96-well polypropylene mother plate from row A to row E, to yield 5 concentrations (50 uM, 10 uM, 2 uM, 400 nM and 80 nM) for each test compound. Row G contains only DMSO. Once titrations are made, the reaction mixture consisting of 30 ng/uL DNA template corresponding to (-160/+379) region on rDNA and 3 mg/mL nuclear extract isolated from HeLa S3 cells in a buffer containing 10 mM Tris HCl pH 8.0, 80 mM KCl, 0.8% polyvinyl alcohol, 10 mg/mL a-amanitin was combined with the test compounds and incubated at ambient for 20 min. Transcription was initiated by addition of rNTP mix (New England Biolabs, Ipswich, MA) to a final concentration of 1 mM and was incubated for one hour at 30 °C. Afterwards DNase I was added and the reaction was further incubated for 2 hr at 37 °C. DNase digestion was terminated by the addition of EDTA to final concentration of 10 mM, followed immediately by 10 min

incubation at 75 °C, and then samples were transferred to 4 °C. The levels of resultant transcript were analyzed by qRT-PCR on 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA) using the following primer-probe set: Pol I probe ctctggcctaccggtgacccggcta, Pol I forward primer gctgacacgctgtcctctggcg and Pol I reverse primer ggctcaagcaggagcgcggc.

Cell viability assay.

We employed cell viability assay that measures metabolic potential of cells by tracking the conversion of resazurin to resofurin. The conversion rate is directly proportional to the number of viable cells and its measurement allows us to determine the effect of drugs on cell proliferation rate and viability. The format of this assay is the same for all cell lines tested.

Assay protocol

3000 cancer cells in 100 uL of cell culture media were plated in each well of a 96-well clear bottom, black wall cell culture-pretreated plate. The next day compounds are serially diluted (3-fold in cell culture media) across a 96-well polypropylene mother plate from row A to row F, to yield 6 concentrations (10 uM, 3.3 uM, 1.1 uM, 370 nM, 124 nM and 41 nM) for each test compound. Rows G and H contain only DMSO. Once titrations are made, the media in plates with cells were disposed and 100 μL of drug dilutions are transferred to plates with cells. After a ninety six-hour incubation at 37 °C, 10 uL of resazurin solution from Alamar Blue Cell Viability kit (Invitrogen, Carlsbad, CA) was added to the media and cells were incubated at 37 °C for three more hours. At the end of this incubation the production of resofurin was measured using Spectrmax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

COMET assay.

To determine if **7c** damages DNA we performed COMET assay.

Assay protocol

A375 cells were treated with **7c** or actinomycin D for 16 hours. The integrity of chromosomal DNA was determined with single cell gel electrophoresis assay (Trevigen, Gaithersburg, MD).

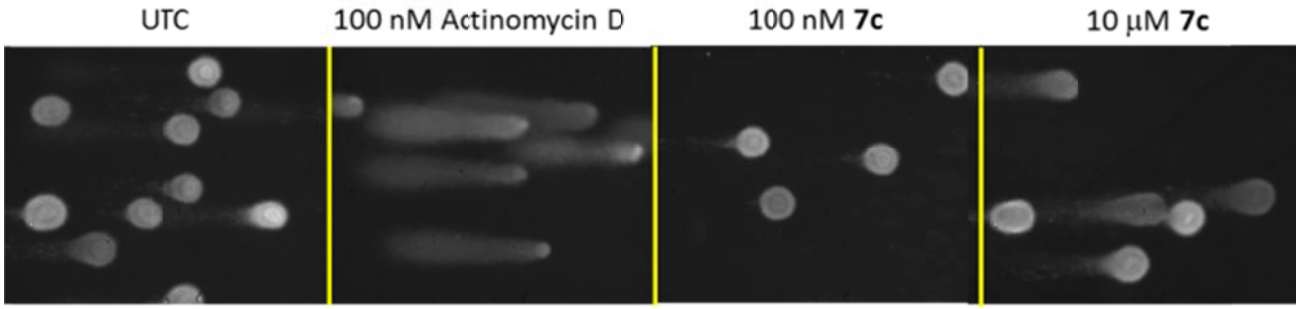


Figure 2. COMMET assay. A375 cells were treated for 16 hours with **7c** or positive control, actinomycin D. The integrity of chromosomal DNA was determined with single cell gel electrophoresis assay (COMET).***DNA Intercalation assay.***

To determine if **7c** can intercalate in DNA we performed DNA intercalation assay.

Assay protocol

7c or Actinomycin-D were serially diluted in DMSO (top concentration 5 mM). In a 96-well plate, drug solution (2 µl) was added to a mixture of Calf Thymus DNA (1.42 mg/ml) and Ehtidium bromide (0.5 µg/ml) in 1.0 M Tris buffer (pH 7.4), total volume of 200 µL. After incubating for 5 minutes at ambient, the fluorescence of the mixture was determined (excitation 260 nM/emission 600 nM).

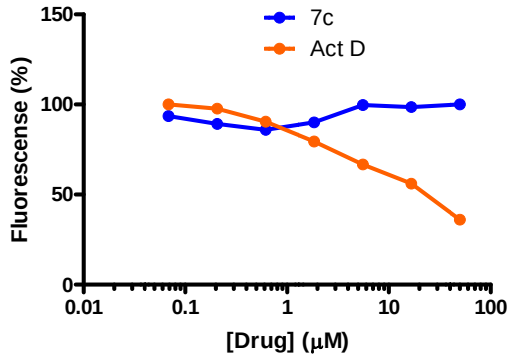


Figure 3. DNA Intercalation Assay. To determine whether **7c** exhibited significant DNA intercalation or minor groove binding, we measured its ability to displace ethidium bromide from Calf Thymus DNA and thus reduce the fluorescence. Known intercalator Actinomycin-D was used as positive control.

In vivo efficacy in murine xenograft model

To determine the in vivo activity of compounds in models of cancer we treated mice xenografts produced from human cancer cells.

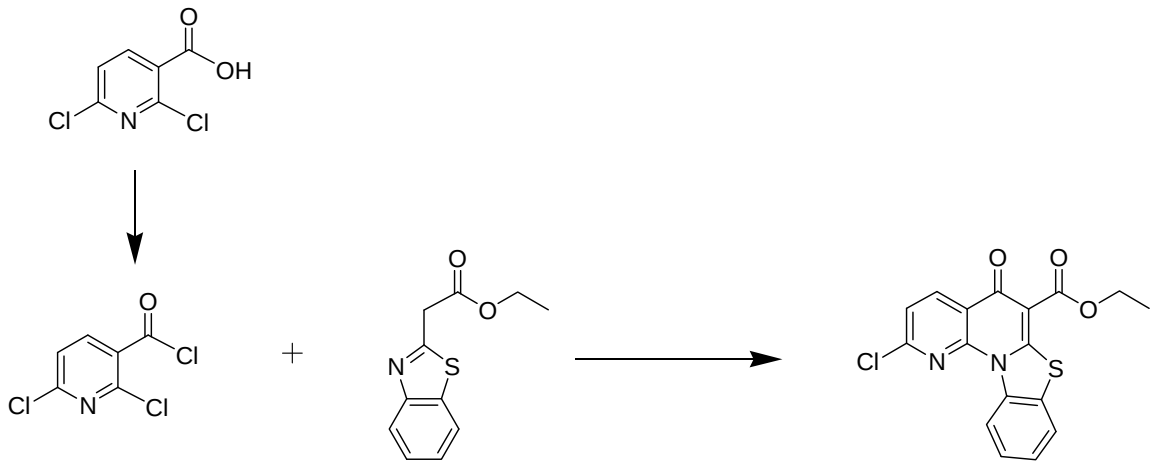
Assay protocol

Animal experiments were performed with five to six week old female athymic (NCr nu/nu fisol) mice of Balb/c origin (Taconic Farms, Germantown, NY) in accordance with approved standard operating protocols of Cylene Pharmaceuticals that were approved by the Institutional Animal Care and Use Committee. Mice were inoculated with $5x10^6$ cells in 100 uL suspension subcutaneouly in the right flank. Tumor measurements were performed by caliper analysis, and tumor volume was calculated using the formula (l * w2)/2, where w = width and l = length in mm of the tumor. Established tumors (~ 110-120 mm^3) were randomized into vehicle (50 mM NaH2PO4 (pH 4.5)), gemcitabine or CX-5461 treatment groups. Tumor Growth Inhibition (TGI) was determined on the last day of study according to the formula: TGI (%) = (100-(VfD- ViD)/ (VfV- ViV)*100), where ViV is the initial mean tumor volume in vehicle-treated group, VfV is the final mean tumor volume in vehicle-treated group ViD is the initial mean tumor volume in drug-treated group and VfD is the final mean tumor volume in drug-treated group.

Preparation and Characterization of CX-5461

Experimental

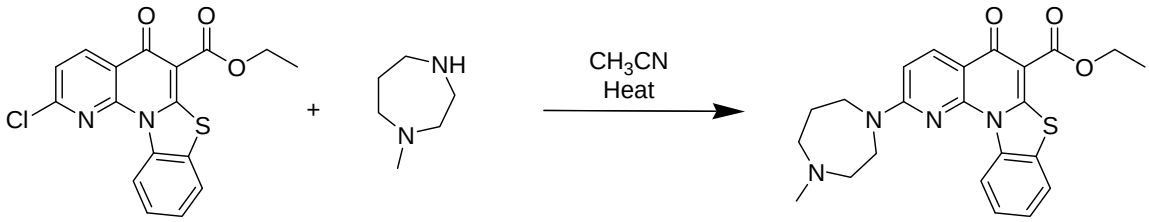
Step1:



To a suspension of 2,6-dichloropyridine-3-carboxylic acid (100 g, 0.52 mol, 1 eq.) and oxalyl chloride (59 mL, 0.68 mol, 1.3 eq.) in DCM (400 mL) was added dropwise DMF (0.4 mL) and the reaction was allowed to stir at room temperature for 18 hours. The resulting solution was filtered and concentrated to a yellow-orange oil and then co-evaporated with ACN to remove excess oxalyl chloride to afford the acid chloride (118 grams) which was used in the next reaction without further purification. To a solution of the benzothiazole ethyl ester (115.24 g, 0.52 mol) in acetonitrile (806.7 mL) at 0 °C was added magnesium chloride (74.39 g, 0.78 mmol, 1.5 eq.) in portions maintaining the temperature below 15 °C. The resulting suspension was chilled to below 5 °C and the dichloronicotinyl chloride (~118 g, 0.52 mol, 1 eq) was added (diluted in 806.7 mL of acetonitrile) *via* addition funnel over 5 minutes. The suspension was then chilled to between -10 °C and 0 °C using a methanol/ice bath and triethylamine (55.33 g, 0.55 mol, 1.06 eq) was added also *via* additional funnel keeping the reaction temperature in the same range. The mixture was stirred for 15 minutes. The cooling bath was removed and water was added (2 X 800 mL, 15 minutes apart) and the suspension was allowed to stir for an additional 15 minutes. The resulting solids were collected by filtration and washed with water (200 mL). The solids were then re-suspended in water (800 mL) for 30 minutes and collected by filtration, washing with water (200 mL). The solids were dissolved in DCM (1.5 L) and the layers were separated. The organic layer was dried over sodium sulfate, filtered and concentrated to dryness, co-evaporating with ACN (200 mL). The resulting solids were suspended in ACN (200 mL) and chilled to below 5 °C for 1 hour. The intermediate product was collected by filtration, washing with cold ACN (150 mL). The intermediate solids were then suspended in ACN (900 mL) and triethylamine (109 mL, 0.78 mol) was added with stirring and the suspension was heated to reflux for 1.5 hours. The mixture was then cooled to below 20 °C and the solids were collected by filtration and washed with ACN (2 X 250 mL). The solids were then stirred with 1.0 L water for 1 hour, filtered and washed with water (2 X 250 mL) then re-suspended in ACN (1 L), stirred for 1 hour, filtered and washed with ACN (2 X 250 mL). The resulting solids were dried under vacuum to afford the chloroester (168.71 g, 0.47 mol, 90.0%) as an

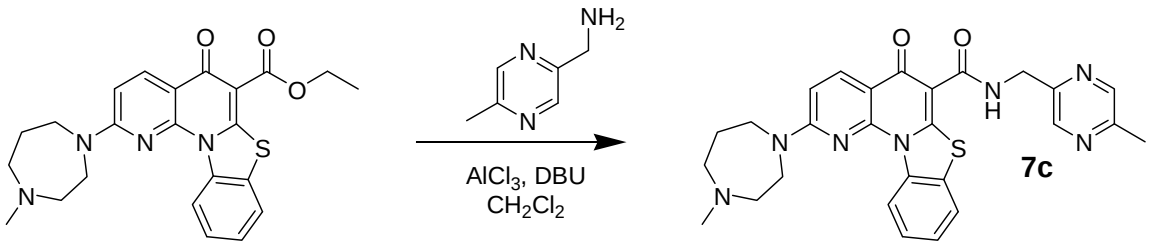
off-white solid. ^{1}HNMR (400 MHz, $CDCl_3$) 1.50 (t, 3H, *J* = 7.2 Hz), 4.52 (q, 2H, *J* = 7.2 Hz), 7.49 (t, 1H, *J* = 7.6 Hz), 7.58 (d, 1H, *J* = 8.4 Hz), 7.61 (t, 1H, *J* = 8.0 Hz), 7.77 (d, 1H, *J* = 8.0 Hz), 8.86 (d, 1H, *J* = 8.0 Hz), 9.55 (d, 1H, *J* = 8.8 Hz); MS (EI): 359.3 $(M+H)^+$.

Step 2:



To a slurry of the chloroester (168.71 g, 470.2 mmol) in acetonitrile (1.68 L) was added N-methylhomopiperazine (112.75 g, 990 mmol) and the mixture was heated to reflux for 4 hours. The mixture was allowed to cool to room temperature then placed in an ice bath until the internal temperature was below 5 °C. The product was collected by filtration and washed with cold (0-5 °C) acetonitrile (2 X 337 mL). The resulting solids were dissolved in methylene chloride (1.6 L) and were washed with a solution of sodium carbonate (26 g in 260 mL water). The layers were separated and the organic layer was dried over sodium sulfate (200 g), filtered and dried under vacuum, co-evaporating with ACN (1 X 500 mL). The resulting solids were suspended in ACN (1.1 L) and chilled to below 5 °C for 1 hour. The resulting solids were collected by filtration, washing with cold ACN (2 X 168 mL) and dried under vacuum to afford the methylhomopiperazine ester as an off-white solid (193.74 g, 440 mmol, 94% yield, 98.7% purity). ^{1}HNMR (400 MHz, $CDCl_3$) 9.51 (d, 1H, *J* = 8.5 Hz), 8.59 (d, 1H, *J* = 8.8 Hz), 7.74 (dd, 1H, *J* = 7.2, 1.2 Hz), 7.48 (dt, 1H, *J* = 7.6, 1.6 Hz), 7.42 (dt, 1H, *J* = 7.6, 0.8 Hz), 7.75 (d, 1H, *J* = 8.8 Hz), 4.51 (q, 2H, *J* = 7.2 Hz), 4.0 (br s, 2H), 3.86 (br s, 2H), 2.90 (br s, 2H), 2.68 (br s, 2H), 2.46 (s, 3H), 2.18 (br m, 2H), 1.7 (br s, 4H), 1.50 (t, 3H *J* = 7.2 Hz). MS (EI): 437.3 $(M+H)^+$.

Step 3:



To a solution of the methylhomopiperazine ester (25.32 g, 58.0 mmol) in DCM (506 mL) was added 2-aminomethyl-5-methylpyrazine (14.3 g, 116.0 mmol) and DBU (26.49 g, 174.0 mmol) and the mixture was chilled to ≤ 0 °C with a methanol/ice bath. Aluminum chloride granules (12.6 g, 94.0 mmol) were added at a rate to maintain the internal temperature between -5 °C and 5 °C the mixture was allowed to stir for 30 minutes. The reaction was quenched with a 50% sodium hydroxide solution (56.0 g) added at a rate to maintain the internal temperature between -5 °C and 5 °C and the resulting brown suspension was allowed to stir, warming to room temperature over 1 hour. The suspension was filtered and washed with DCM (100 mL). The combined filtrates were washed with brine (100 mL), dried over sodium sulfate, treated with charcoal and filtered. The solvent was removed under vacuum and co-evaporated with methanol (100 mL) and the resulting solid was triturated twice from methanol (633 mL) overnight at room temperature. The resulting suspension was chilled to below 5 °C and the solids were collected by filtration, washing with cold methanol (below 5 °C, 2 X 50 mL). The resulting solids were dried under vacuum to afford the product as an off-white solid (24.1 g, 47.0 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.26 (1H, t, *J* = 5.2 Hz), 9.37 (1H, d, *J* = 8.4 Hz), 8.59 (1H, d *J* = 1.6 Hz), 8.45 (1H, d, *J* = 4.4 Hz), 8.42 (1H, s), 7.67 (1H, dd, *J* = 7.2, 1.6 Hz), 7.37 (2H, m), 6.67 (1H, d, *J* = 9.2 Hz), 4.85, (2H, d, *J* = 6.0 Hz), 3.87 (2H, br s), 3.78 (2H, br s), 2.80 (2H, br t, *J* = 4.0 Hz), 2.60 (2H, br t, *J* = 4.8 Hz), 2.55 (3H, s), 2.40 (3H, s), 2.09 (3H, m) ppm. MS (EI): 514.3 $(M+H)^+$.

NMR Characterization of Compounds in table 2

Compound **2**

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.57 (1H, t, *J* = 4.8 Hz), 9.44 (1H, d , *J* = 8.0 Hz), 8.61 (1H, d, *J* = 8.8 Hz), 7.75 (1H, dd, *J* = 7.2, 1.2 Hz), 7.49 (1H, dt, *J* = 7.6, 1.2 Hz), 7.43 (1H, dt, *J* = 7.6, 1.2 Hz), 6.92 (1H, d, *J* = 8.8 Hz), 3.93 (4H, brt, *J* = 4.5 Hz), 3.81 (4H, brt, *J* = 4.8 Hz), 3.56 (2H, m), 3.16 (1H, brt, *J* = 8.4 Hz), 2.40 (3H, s), 2.25 (2H. brm), 2.13 (2H, brm), 1.84 (2H, brm), 1.74 (2H brm).

Compound **1a**

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.60 (1H, t, *J* = 5.6 Hz), 9.42 (1H, d , *J* = 8.0 Hz), 8.60 (1H, d, *J* = 8.8 Hz), 7.74 (1H, dd, *J* = 7.6, 1.2 Hz), 7.47 (1H, dt, *J* = 6.8, 1.2 Hz), 7.41 (1H, dt, *J* = 7.6, 1.2 Hz), 6.89 (1H, d, *J* = 9.6 Hz), 3.93 (4H, brt, *J* = 5.6 Hz), 3.80 (4H, brt, *J* = 4.4 Hz), 3.67 (2H, q, *J* = 6.8 Hz), 2.78 (2H, t, *J* = 6.8 Hz), 2.62 (4H, brt, *J* = 5.6 Hz), 1.81 (4H, m, *J* = 3.6 Hz).

Compound **2a**

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.52 (1H, t, *J* = 5.6 Hz), 9.41 (1H, d , *J* = 7.6 Hz), 8.30 (1H, d, *J* = 12.8 Hz), 7.75 (1H, dd, *J* = 7.2, 1.2 Hz), 7.49 (1H, dt, *J* = 7.6, 1.2 Hz), 7.44 (1H, dt, *J* = 7.2, 1.2 Hz), 3.96 (4H, brt, *J* = 4.4 Hz), 3.84 (4H, brt, *J* = 4.4 Hz) 3.69 (2H, q, *J* = 6.0 Hz), 2.81 (2H, t, *J* = 6.8 Hz), 2.67 (4H, brs), 1.83 (4H, m, *J* = 3.2 Hz).

Compound **3b**

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.36 (1H, d, *J* = 8.4 Hz), 8.29 (1H, d, *J* = 12.8 Hz), 7.65 (1H, dd, *J* = 8, 1.6 Hz), 7.48 (1H, dt, *J* = 7.6, 1.6 Hz), 7.42 (1H, dt, *J* = 7.6, 1.2 Hz), 3.95 (4H, brt, *J* = 4.8 Hz), 3.88 (2H, brs), 3.81 (4H, brt, *J* = 4.8 Hz), 3.48 (2H, brt, *J* = 4.4 Hz), 2.54 (2H, Brt, *J* = 5.2 Hz), 2.45 (2H, brs), 2.32 (3H, s).

Compound **4a**

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.53 (1H, t, *J* = 5.6 Hz), 9.24 (1H, dd , *J* = 11.2, 2.4 Hz), 8.60 (1H, d, *J* = 8.8 Hz), 7.67 (1H, dd, *J* = 8.4, 1.2 Hz), 7.49 (1H, dt, *J* = 7.6, 1.2 Hz), 7.19 (1H, dt, *J* = 8.8, 2.4 Hz),

6.91 (1H, d, *J* = 9.2 Hz), 3.94 (4H, brt, *J* = 5.6 Hz), 3.80 (4H, brt, *J* = 5.2 Hz) 3.67 (2H, q, *J* = 7.2 Hz), 2.77 (2H, t, *J* = 6.8 Hz), 2.62 (4H, brt, *J* = 5.6 Hz), 1.81 (4H, m, *J* = 3.6 Hz).

Compound **5c**

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.21 (1H, t, *J* = 6.0 Hz), 9.44 (1H, d , *J* = 7.6 Hz), 8.62 (1H, d, *J* = 9.2 Hz), 8.57 (1H, d, *J* = 1.2 Hz), 8.45 (1H, d, *J* = 1.2 Hz), 7.75 (1H, dd, *J* = 7.6, 1.2 Hz), 7.49 (1H, dt, *J* = 7.6, 1.2 Hz), 7.43 (1H, dt, *J* = 7.6, 1.2 Hz), 6.91 (1H, d, *J* = 8.8 Hz), 4.86 (1H, d, *J* = 6.0 Hz), 3.93 (4H, brt, *J* = 4.8 Hz), 3.81 (4H, brt, *J* = 5.2 Hz) 2.55 (3H, s).

Compound **6c**

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.22 (1H, t, *J* = 5.6 Hz), 9.44 (1H, d , *J* = 7.6 Hz), 8.57 (1H, d, *J* = 2.8 Hz), 8.55 (1H,s), 8.44 (1H, s), 7.73 (1H, dd, *J* = 8.0, 1.6 Hz), 7.46 (1H, dt, *J* = 6.8, 1.6 Hz), 7.41 (1H, dt, *J* = 7.2, 0.8 Hz), 6.90 (1H, d, *J* = 9.2 Hz), 4.85 (1H, d, *J* = 5.6 Hz), 3.84 (4H, brt, *J* = 4.4 Hz), 2.60 (4H, brt, *J* = 5.2 Hz), 2.54 (3H, s), 2.39 (3H, s).

Compound **8c**

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.26 (1H, t, *J* = 5.6 Hz), 9.49 (1H, d , *J* = 8.4 Hz), 8.60 (1H, d, *J* = 8.8 Hz), 8.57 (1H,d, *J* = 1.2 Hz), 8.45 (1H, d, *J* = 1.2Hz), 7.76 (1H, dd, *J* = 7.2, 1.2 Hz), 7.47 (1H, dt, *J* = 6.8, 1.6 Hz), 7.43 (1H, dt, *J* = 7.6, 1.2 Hz), 6.83 (1H, d, *J* = 8.8 Hz), 4.86 (1H, d, *J* = 5.6 Hz), 3.99 (6H, brs), 3.79 (2H, t, *J* = 5.6 Hz), 2.55 (3H, s), 2.16 (2H, brm).

Compound **9c**

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.26 (1H, t, *J* = 5.6 Hz), 9.52 (1H, d , *J* = 8.4 Hz), 8.57 (1H, d, *J* = 1.6 Hz), 8.57 (1H,d, *J* = 9.2 Hz), 8.45 (1H, d, *J* = 1.6Hz), 7.75 (1H, dd, *J* = 7.2, 1.6 Hz), 7.47 (1H, dt, *J* = 7.2, 1.6 Hz), 7.43 (1H, dt, *J* = 7.6, 1.2 Hz), 6.80 (1H, d, *J* = 9.6 Hz), 4.86 (1H, d, *J* = 6.0 Hz), 4.04 (2H, brs), 3.88 (2H, brs), 2.99 (2H, brs), 2.75 (2H, brs), 2.68 (2H, brs), 2.55 (3H, s), 2.20 (2H, brm), 1.15 (3H, brs).

Compound **10c**

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.29 (1H, t, *J* = 5.6 Hz), 9.56 (1H, d , *J* = 8.0 Hz), 8.57 (1H,s), 8.54 (1H,d, *J* = 9.2 Hz), 8.45 (1H, s), 7.75 (1H, dd, *J* = 7.6, 1.2 Hz), 7.47 (1H, dt, *J* = 6.8, 1.2 Hz), 7.42 (1H, dt, *J* = 7.6, 1.2 Hz), 6.79 (1H, d, *J* = 9.2 Hz), 4.86 (2H, d, *J* = 5.2 Hz), 3.90 (4H, brs), 2.99 (2H, brs), 2.72 (2H, brs), 2.55 (3H, s), 2.02 (1H, brm), 1.77 (2H, brm), 1.66 (10H, brm).